Published in final edited form as:

Science. 2010 February 12; 327(5967): 869–872. doi:10.1126/science.1183218.

Darwinian Evolution of Prions in Cell Culture*

Jiali Li^{1,*}, Shawn Browning^{1,*}, Sukhvir P. Mahal¹, Anja M. Oelschlegel¹, and Charles Weissmann^{1,†}

¹ Department of Infectology, Scripps Florida, 130 Scripps Way, Jupiter, FL 33458, USA

Abstract

Prions are infectious proteins consisting mainly of PrP^{Sc}, a β sheet-rich conformer of the normal host protein PrP^C, and occur in different strains. Strain identity is thought to be encoded by PrP^{Sc} conformation. We found that biologically cloned prion populations gradually became heterogeneous by accumulating “mutants”, and selective pressures resulted in the emergence of different mutants as major constituents of the evolving population. Thus, when transferred from brain to cultured cells, “cell-adapted” prions outcompeted their “brain-adapted” counterparts, and the opposite occurred when prions were returned from cells to brain. Similarly, the inhibitor swainsonine selected for a resistant substrain, while in its absence the susceptible substrain outgrew its resistant counterpart. Prions, albeit devoid of a nucleic acid genome, are thus subject to mutation and selective amplification.

Prions are the infectious agents responsible for a variety of neurodegenerative disorders including scrapie in sheep, bovine spongiform encephalopathy (BSE) in cattle, and new variant Creutzfeldt-Jacob disease (CJD) and kuru in humans. The principal, if not only component of the prion is PrP^{Sc}, a beta-sheet-rich conformer of prion protein (PrP). PrP^{Sc} propagates by eliciting conversion of PrP^C, the physiological form of PrP, into a likeness of itself. The seeding hypothesis posits that PrP^C is in equilibrium with PrP^{Sc} or a PrP^{Sc} precursor, with the equilibrium largely in favor of PrP^C, and that PrP^{Sc} is only stabilized when it forms an aggregate, or seed, containing a critical number of monomers. Once a seed is present, monomer addition ensues rapidly (1).

Prions occur in the form of distinct strains, originally characterized by the incubation time and the neuropathology they elicit in a particular host (2). Many different strains can be propagated indefinitely in hosts homozygous for the PrP gene (*Prnp*); the protein-only hypothesis assumes each strain is associated with a different conformer of PrP^{Sc} (3–5), implying that there are as many stable conformations of PrP as there are stable prion strains that can be propagated in a particular mouse strain, perhaps fifteen or more (6). The concept of “conformation templating” at the protein level was first supported by cell-free conversion experiments (7) and extended by the development of protein misfolding cyclic amplification (PMCA), which mimics PrP^{Sc} autocatalytic replication in vitro (8).

A prion strain, transferred from one species to another and subsequently returned to the original host, may in some instances have changed or “mutated” (9,10). Novel strains may arise not

* **Publisher's Disclaimer:** This manuscript has been accepted for publication in *Science*. This version has not undergone final editing. Please refer to the complete version of record at <http://www.sciencemag.org/>. This manuscript may not be reproduced or used in any manner that does not fall within the fair use provisions of the Copyright Act without the prior, written permission of AAAS.

†To whom correspondence should be addressed. charlesw@scripps.edu.

*These authors contributed equally to this work.

Note added in proof: Exposure of mice or differentiated neuroblastoma cells infected with RML prions to quinacrine leads to drug-resistant prions (32).

only by mutation of naturally occurring strains, but also de novo, in transgenic mice (11) or in cell-free systems, mediated by PMCA (12).

Strains are classically differentiated by mouse bioassays that require months or years to complete. The Cell Panel Assay (CPA) (13) allows assessment of the characteristic cell tropism of strains by the Standard Scrapie Cell Assay (SSCA) (14) on a panel of 4 cell lines, the neuroblastoma-derived PK1 and R33, the neuronal CAD and the fibroblastic LD9 lines. The CPA readily distinguishes between RML, 22L, ME7 and 301C prions within about 2 weeks. We have found that 2 $\mu\text{g/ml}$ (11.55 μM) swainsonine (swa), an inhibitor of Golgi α -mannosidase II that impairs formation of complex N-linked glycans, inhibits by 99% or more chronic infection of PK1 cells by RML and 79A but not by 22L prions. The ED_{50} for inhibition of infection is 3 ng/ml. The misglycosylation of host proteins caused by swa has no effect on cell growth but reduces the accumulation rate of “swa-sensitive”, but not of “swa-resistant” prions below the replication rate of the host cells, causing the prions to be diluted out with progressive cell doublings.

It has been reported that strain specificity is retained when prions are transferred from brain to cultured cells and back to brain (15,16), however the properties of the prions while in cell culture could not be determined by classical procedures. We therefore examined this question using the CPA. We generated a chronically infected cell population, PK1[22L]_{wp}, by exposing PK1 cells to 22L-infected mouse brain homogenate (brain[22L]) and propagating them for about 34 doublings. The CPA showed that cell-derived and brain-derived prions differed: the cell-derived prions were unable to infect R33 cells (“R33 incompetent”) or to infect PK1 cells in the presence of swa (“swa sensitive”), in contrast to brain[22L]-derived prions, which were able to do both. (Fig. 1A,C). We considered that cell- or brain-derived components might influence the infectious properties of the prions on the cell panel. If this were so, the full change in properties should be observed immediately after the first round of prion replication in the cells. We therefore infected PK1 cells with 22L-infected brain homogenate, collected conditioned medium 9 days after infection (P0) and after successive 1:10 splits. The CPA (Fig. 1B) showed that at P0 the secreted prions resembled brain prions, in that they were R33-competent and swa-resistant, and that with successive splits they became less infectious to R33 and more susceptible to inhibition by swa. By the 12th 1:10 split, i.e. after about 40 doublings, the properties of the population were indistinguishable from those of the PK1[22L]_{wp}. Thus, upon transfer from brain to PK1 cells the prion population underwent a gradual, not a sudden change in properties. This suggested that the brain-derived population might be heterogeneous, and that the R33-competent, swa-resistant prions that predominated in brain were replaced by R33-incompetent, swa-sensitive prions with a growth advantage in PK1 cells.

Having established that the prions in PK1[22L]_{wp} cells were swa sensitive, we attempted to cure the cells of infection by propagating them in the presence of swa for ten 1:20 splits (Fig. 2A,B). In the absence of swa, the percentage of PrP^{Sc}-positive cells remained essentially unchanged, between 30 and 40%. In the presence of swa the percentage initially dropped, from 35% to about 7%, but then, unexpectedly, increased to reach a value of about 25% by the 8th – 10th split, suggesting the development of resistance. To investigate this in more detail, we analyzed the swa susceptibility of prions secreted by PK1[22L]_{wp} cells. Prions from cells propagated in the absence of swa were swa sensitive at all splits tested (Fig. 2C). In contrast, prions from cells propagated in the presence of swa for 2 or more splits were completely swa resistant. Remarkably, PK1[22L]_{wp} cells grown for 5 passages in the presence of swa and then for 5 splits in its absence secreted prions that were again fully swa sensitive. Thus, in the presence of the drug, pre-existing, or newly generated swa-resistant prion variants selectively grew to dominate the population. Moreover, after withdrawal of the inhibitor, residual or newly generated swa-sensitive prions replaced their swa-resistant counterparts almost completely after about 22 doublings, presumably because in the absence of swa the drug-sensitive prions

replicated more rapidly. We confirmed all these results by assaying lysates of the same cell cultures described above (17). Moreover, the entire experiment was repeated by a different operator with the same results.

Swa prevents normal complex glycosylation of N-linked glycans (18) and the resulting high-mannose glycans are cleavable by endoglycosidase H (Endo H), in contrast to native complex glycans, which are completely resistant. Treatment of proteinase K (PK)-digested samples from control PK1[22L]_{wp} cells (Fig. 2D) with Endo H did not result in PrP bands with increased mobility; however, already after the first 1:20 split (about 4.3 doublings) in swa-containing medium (Fig. 2D), there was a significant mobility shift of all bands due to the loss of complex glycosylation, and Endo H treatment caused a dramatic increase in mobility. Yet, as described above (Fig. 2C, table S1), the prions from this sample were still swa sensitive, showing that the lack of complex glycosylation was not the cause of swa resistance. When swa treatment was discontinued, the mobility pattern reverted to that of untreated controls after two 1:20 splits (Fig. 2D, “SC7”) and the glycans were completely resistant to Endo H. Strikingly, however, the prions continued to be swa-resistant until after the 5th split (SC10). Thus, lack of complex glycosylation is not responsible for swa-resistance, and normally glycosylated PrP^{Sc} can be associated with both swa-sensitive and swa-resistant prion variants.

Some prion strains differ in the site at which the cognate PrP^{Sc} is cleaved by PK (3,4,19) or thermolysin (20) or in the rate at which it is degraded (21). We treated lysates of swa-sensitive and swa-resistant PK1[22L]_{wp} cells with PK or thermolysin (fig. S3) and detected no significant differences in the electrophoretic mobility of the cleavage products. There was no significant difference in the susceptibility of PrP^{Sc} from brain[22L], swa-sensitive or swa-resistant PK1[22L]_{wp} cells to PK (fig. S4). PrP^{Sc} from different strains may exhibit different stabilities, as determined by susceptibility to PK digestion after exposure to increasing concentrations of guanidinium chloride (Gnd.HCl) (11,22). We subjected lysates of swa-sensitive and swa-resistant PK1[22L]_{wp} cells as well as homogenates of brain[22L] to the conformational stability assay, but found no significant differences in the Gnd.HCl_{1/2} values, which were 1.1–1.3 M in all cases (fig. S5). These negative findings suggest that structural differences between the postulated sub-strains may be discrete.

Cell lysates were injected intracerebrally (i.c.) into C57BL/6 mice and brains were recovered when the mice became terminally ill at 147 days after inoculation (table S2). The CPA of authentic brain[22L], the brain-passaged swa-sensitive and swa-resistant PK1[22L]_{wp} prions gave indistinguishable patterns (Fig. 1C, S6), showing that the PK1-derived 22L prions regained their original cell tropism after propagation in brain. Brain sections revealed the vacuolization of the granular layer of the cerebellum and the loss of Purkinje cells typical for 22L for both cell-derived (swa-sensitive and swa-resistant) and brain-derived samples (fig. S7, table S3).

The finding that exposure of 22L-infected PK1 cells to swa leads to the emergence of swa-resistant prions means that such variants either exist in the population at a low level prior to exposure to swa or that they are generated during exposure to the drug. To address this question we exposed PK1 cells to PK1[22L]_{wp} prions either in the presence or absence of swa for 2 days, distributed the cells into 96-well plates at 8 cells/well for the cells infected in the absence of swa and 1000 cells/well for cells infected in the presence of swa (fig. S2). Uninfected cells were added to bring the total number of cells in each well to 1000. The cells were grown to confluence and split 1:10 five times, in the continued absence or presence of swa. Under these conditions, any well containing one or more infected cells yields a positive signal in the PK-ELISA (17) because of the continuous spread of infection (14). The average number of infected cells delivered to each well was calculated by the Poisson equation. 0.029% of the wells from the swa-exposed population and 5.8% from the non-swa-exposed population were PrP^{Sc}.

positive (fig. S2). Because swa inhibits replication of PK1[22L]_{wp} prions by more than 99%, these results indicate that 0.5% of the prions secreted by PK1[22L]_{wp} cells were swa-resistant before exposure to the drug.

The 22L isolate obtained from the TSE Resource Center, Compton, had been biologically cloned twice in succession (23), yet here we found it to be heterogeneous with regard to its swa sensitivity after transfer to cultured cells, suggesting that variants had arisen during the two rounds of propagation in mice and the transfer to cells. To ascertain whether cloned prion populations could become heterogeneous, we recloned 22L prions by endpoint dilution in cell culture (16) and analyzed the populations after various extents of propagation (Fig. 3). Eight clonal populations were assayed after about 31 doublings following infection and found to secrete swa-sensitive prions (fig. S8). Aliquots of each population were propagated for 22 doublings in the presence or absence of swa; 2 of the 8 clones, 8C4 and 3C6, secreted swa-resistant prions after being grown in the presence, but not in the absence of the drug (Fig. 3A). The other six cell clones secreted swa-sensitive prions (Fig. 3A), and lost infectivity after being propagated for five 1:20 splits in the presence of swa (Fig. 3A). Three of the six clones (8A8, 8B4, 8H6) were propagated for additional 22 doublings in the absence of the drug ((b) to (c1)), altogether about 53 doublings) and were then exposed for 22 doublings to swa, whereupon one more clone (8A8) produced swa-resistant prions (Fig. 3A). Thus, swa-resistant variants arose during propagation of cloned, swa-sensitive prion populations for as few as 31 doublings in the absence of the drug. From these data we calculated (17) a very approximate “mutation rate” of 10^{-6} /doubling. This number is an underestimate, mainly because we considered neither the selective disadvantage of swa-resistant prions in the absence of swa nor the “reversion rate”.

Perhaps swa-resistance and R33-competence are only two of many variations that can arise in a 22L population; if so, the overall mutation rate could be even greater and the prion population more diverse, comprising a multiplicity of “substrains” or “types” (24). This would be reminiscent of the “quasispecies” concept (25,26). Whilst heterogeneity in the case of RNA viruses is due to point mutations resulting from error-prone replication, heterogeneity in the case of the prions are likely to be due to differences in the structure (other than the amino acid sequence) of the PrP^{Sc} molecules. These differences could reflect variations in the conformation of the PrP^{Sc} resulting during conversion; the conformational changes may be subtle, but sufficient to facilitate propagation in a particular environment. Alternatively, or in addition, variation could be due to cell-derived determinants, for example association of PrP with a small cellular RNA, or to the nature of its glycosylation.

Transfer of a prion strain from one animal species to another usually entails a low attack rate and long incubation times, which in subsequent passages are dramatically reduced (27). If the only barrier to prion transfer between species were the initial round of heterologous conversion, then once it occurred, propagation should be rapid. However, this is almost never so, and two or three sequential transmissions are required to obtain stable, shortened incubation times, suggesting that additional, likely conformational, changes are required to optimize prions for propagation in the new host (9,10,28). It has also been argued that even within a single host, different “strain types” may develop within different tissues (29–31), and the “cloud” model to explain these observations (24) is well supported by our findings.

Wherein do the concepts of strains and substrains differ? The energy landscape diagram of fig. S10 depicts the view that substrains are distinct collectives of prions that can interconvert reproducibly and relatively readily, that is, within the generation time of the host or a few dozen rounds of replication, because they are separated by relatively low activation energy barriers; strains, each comprising a set of readily interconvertible substrains, are separated by higher energy barriers, causing transitions to be rare events.

In summary, prions show the hallmarks of Darwinian evolution: They are subject to mutation, as evidenced by heritable changes of their phenotypic properties, and to selective amplification, as documented by the emergence of distinct populations in different environments. A practical consequence of our findings is the realization that therapeutic approaches aimed at stabilizing PrP or reducing PrP expression are less likely to be thwarted by emergence of drug resistance than those based on targeting PrP^{Sc}.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

References and Notes

1. Jarrett JT, Lansbury PJ. Cell 1993;73:1055. [PubMed: 8513491]
2. Bruce, ME.; Fraser, H.; McBride, PA.; Scott, JR.; Dickinson, AG. Prion Diseases of Humans and Animals. Prusiner, SB.; Collinge, J.; Powell, J.; Anderton, B., editors. Ellis Horwood; New York, London: 1992. p. 497-508.
3. Bessen RA, Marsh RF. J Virol 1992;66:2096. [PubMed: 1347795]
4. Telling GC, et al. Science 1996;274:2079. [PubMed: 8953038]
5. Peretz D, et al. Protein Sci 2001;10:854. [PubMed: 11274476]
6. Bruce ME, Dickinson AG. J Gen Virol 1987;68:79. [PubMed: 3100717]
7. Caughey B, et al. Mol Biotechnol 1999;13:45. [PubMed: 10934521]
8. Saborio GP, Permanne B, Soto C. Nature 2001;411:810. [PubMed: 11459061]
9. Kimberlin RH, Cole S, Walker CA. J Gen Virol 1987;68:1875. [PubMed: 3110370]
10. Kimberlin RH, Walker CA, Fraser H. J Gen Virol 1989;70:2017. [PubMed: 2504883]
11. Legname G, et al. Proc Natl Acad Sci U S A 2006;103:19105. [PubMed: 17142317]
12. Barria MA, Mukherjee A, Gonzalez-Romero D, Morales R, Soto C. PLoS Pathog 2009;5:e1000421. [PubMed: 19436715]
13. Mahal SP, et al. Proc Natl Acad Sci U S A 2007;104:20908. [PubMed: 18077360]
14. Klohn PC, Stoltze L, Flechsig E, Enari M, Weissmann C. Proc Natl Acad Sci U S A 2003;100:11666. [PubMed: 14504404]
15. Arjona A, Simarro L, Islinger F, Nishida N, Manuelidis L. Proc Natl Acad Sci U S A 2004;101:8768. [PubMed: 15161970]
16. Arima K, et al. J Virol 2005;79:7104. [PubMed: 15890950]
17. Materials and methods, as well as additional experiments, are available as supporting material on Science Online.
18. Tulsiani DR, Touster O. J Biol Chem 1983;258:7578. [PubMed: 6408079]
19. Collinge J, Sidle KC, Meads J, Ironside J, Hill AF. Nature 1996;383:685. [PubMed: 8878476]
20. Owen JP, et al. J Virol 2007;81:10532. [PubMed: 17652380]
21. Kuczius T, Groschup MH. Mol Med 1999;5:406. [PubMed: 10415165]
22. Peretz D, et al. Neuron 2002;34:921. [PubMed: 12086640]
23. McConnell, I.; Barron, RM. personal communication
24. Collinge J, Clarke AR. Science 2007;318:930. [PubMed: 17991853]
25. Eigen M. Trends Microbiol 1996;4:216. [PubMed: 8795155]
26. Epstein IR, Eigen M. Biophys Chem 1979;10:153. [PubMed: 486701]
27. Bruce ME, Fraser H. Curr Top Microbiol Immunol 1991;172:125. [PubMed: 1810707]
28. Kellershohn N, Laurent M. Biochem J 1998;334:539. [PubMed: 9729459]
29. Fraser, H.; Bruce, ME.; Davies, D.; Farquhar, CF.; McBride, PA. Prion Diseases of Humans and Animals. Prusiner, SB.; Collinge, J.; Powell, J.; Anderton, B., editors. Horwood; London: 1992. p. 59-61.
30. Hilton DA, Fathers E, Edwards P, Ironside JW, Zajicek J. Lancet 1998;352:703. [PubMed: 9728989]

31. Wadsworth JD, et al. Lancet 2001;358:171. [PubMed: 11476832]
32. Ghaemmaghami S, et al. PLoS Pathog 2009;5:e1000673. [PubMed: 19956709]
33. We thank C.A. Demczyk for assistance with cell culture, E. Smith for assistance with the SSCA, Ira Suponitsky-Kroyter for the proteolytic digestion analyses and A. Sherman for histochemistry and animal work. We thank T. Bartfai, G. Joyce and C. Lasmezas for critical reading of the manuscript and valuable suggestions. The project was supported by an RO1 grant (#NS059543) from the NIH and by a generous donation from the Alafi Family Foundation to C.W..

Fig. 1.

Prion characterization by the CPA. Cells (LD9, violet; CAD, brown; R33, green; PK1, light blue; PK1 + 12 μ M swa, red) were exposed to the serially diluted samples indicated. The proportion of PrP^{Sc}-positive cells was plotted against log₁₀ dilution. (A) Brain[22L]-derived prions are swa resistant and R33 competent while those from 22L-infected PK1 cells (about 27 doublings after infection) are swa sensitive and R33 incompetent. (B) Transfer of prions from brain to PK1. PK1 cells were exposed to 10⁻⁴ brain[22L] for 1 day and propagated 9 days (P0); further propagation for 12 1:10 splits yielded P1 to P12. Conditioned media were analyzed on PK1 cells with (red) or without (blue) 2 μ g/ml swa, and on R33 cells (green). Infection in presence of 10 μ g/ml pentosan polysulfate, which abolishes prion replication, yielded no infectivity, documenting absence of inoculum. (C) Brain homogenates from terminal C57BL/6 mice inoculated with lysates of PK1[22L]_{wp} cells propagated in absence (a) or presence (b)

of swa or with (c) 1% 22L-infected brain homogenate gave the same CPA responses. (d) The very different CPA response of brain RML is shown for comparison.

Fig. 2.

Propagation of 22L-infected PK1 cells in swa results in swa-resistant prions. **(A)** Scheme. PK1 [22L]_{wp} cells were either grown in swa (2 µg/ml) for up to 10 splits “S1-10”, without swa for up to 10 splits “C0-10”, or with swa for 5 splits followed by without swa for 5 splits “SC6-10”. **(B)** The level of PrP^{Sc}-positive cells propagated without swa (blue), as assessed by PK-ELISA. **(C)** Swa susceptibility of secreted prions propagated in the presence or absence of swa. 100× concentrated conditioned medium was assayed on PK1 cells in the presence (red) or absence (blue) of swa. **(D)** Swa-resistant prions are associated both with PrP^{Sc} carrying high-mannose glycans (S2-S5) and normally glycosylated PrP^{Sc} (SC7- SC8). PK1[22L]_{wp} cell lysates digested with PK and Endo H were subjected to immuno blotting with anti-PrP. After the first

split in swa PrP bands shift to higher mobility reflecting inhibition of complex glycosylation, and Endo H digestion results in a large mobility increase due to removal of high-mannose glycans. After 2 splits without swa (SC7), normal glycosylation is restored but swa resistance is retained.

Fig. 3.

Development of heterogeneity in cloned prion populations. Prions were cloned by end point dilution in cell culture and eight clones were tested for their ability to yield swa-resistant populations after propagation in swa. Values in parentheses indicate number of doublings. (A) PK1 cells were exposed to PK1[22L]_{wp} conditioned medium for 2 days, leading to infection of about 4% of the cells. Cells were distributed at 0.3, 1, 3, or 8 cells/well (a), along with about 1000 uninfected cells, grown to confluence (7 doublings) and split 1:10 five times (17 doublings) in 96-well plates. 23 of 602 wells scored positive by PK-ELISA. Eight clones were expanded for 7 doublings and all secreted swa-sensitive prions (b, altogether 31 doublings). After propagation in swa for five 1:20 splits, clones 8C4 and 3C6 yielded swa-resistant prions

(c2), while the others were cured (c3). Propagation without swa yielded swa-sensitive prions (c1). Three of 6 clones that failed to yield swa-resistant populations after exposure to swa (8A8, 8B4, 8H6) were further passaged for 22 doublings without swa (c1) followed by 22 doublings with swa, whereupon one (8A8) yielded swa-resistant prions (d2) and two (8B4, 8H6) were cured (d3). Details in (17). (B) Swa-resistance was determined by assaying 100× concentrated conditioned medium on PK1 cells with or without swa.